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Regulation of the protein stability of EMT transcription factors

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The epithelial to mesenchymal transition (EMT) consists of a rapid change of cell phenotype, characterized by the loss of epithelial characteristics and the acquisition of a more invasive phenotype. Transcription factors regulating EMT (Snail, Twist and Zeb) are extremely labile proteins, rapidly degraded by the proteasome system. In this review we analyze the current mechanisms controlling degradation of EMT transcription factors, focusing on the role of new E3 ubiquitin-ligases involved in EMT. We also summarize the regulation of the stability of these EMT transcription factors, specially observed in different stress conditions, such as hypoxia, chemotherapeutic drugs, oxidative stress or γ-irradiation.

EMT and Transcription Factors Driving EMT

Epithelial to mesenchymal transition (EMT) is a reversible process that promotes epithelial cells to acquire a mesenchymal phenotype. During this transition the cell-cell junction structures, including adherens junctions and desmosomes, are disassembled and cells become spindle-shaped and motile. EMT was identified in the context of development decades ago[1] and has been extensively reviewed.[2] However, it was not until the discovery of its role in tumor invasion that its molecular mechanisms have started to be unraveled.[3]

Although examples of EMT have been characterized in development, for instance during gastrulation and neural crest delamination,[4-6] EMT is also relevant in tumor progression. Expression of EMT markers in primary tumors correlates with enhanced invasiveness and poor clinical prognosis.[7] Moreover, lineage tracing experiments have recently demonstrated the occurrence of EMT in pancreas tumors and its involvement in tumor invasion.[8] It has also been proposed that cancer cells having undergone EMT and located at the tumor edge secrete cytokines and proteases that promote angiogenesis, remodel the peritumoral extracellular matrix (ECM) and activate non-neoplastic cells.[9] Simultaneously, stromal cells release factors that increase EMT in cancer cells, fostering survival, growth and invasiveness of the tumor, generating a reciprocal influence between the tumor and its microenvironment.[9-11] Mesenchymal cells resulting from EMT are more resistant to cell death and senescence, as well as escape immune surveillance and thus avoid chemo- and immuno-therapies.[4]

In recent years the concept of EMT has been modified and refined including the term of "partial EMT."[12,13] This refers to a phenotype often observed *in vivo* in the processes of wound healing and mammary tubulogenesis. Partial EMT, as the name suggests, is an intermediate process in which some of the characteristics of epithelial cells are retained while there is a simultaneous and evident induction of mesenchymal features. This phenotype allows cells to spread and migrate in an active manner although a cell-cell cohesiveness is maintained to a certain extent and cells present a "cohort-like" migration; it is likely that this partial EMT is more suitable to be reversed than a complete EMT and therefore more capable to sustain the growth of secondary tumors or metastasis.[14,15]

The key biomarker for EMT is the down-regulation of the homotypic adherens junction protein E-cadherin.[16] E-cadherin downregulation happens mainly due to transcription inhibition, through the action of different transcription factors on consensus E-boxes (5′-CACCTG-3′ or 5′-CAGGTG-3′) present in the E-cadherin (CDH1) promoter.[17,18] Among the plethora of transcription factors repressing E-cadherin, only the members of the Snail family Snail1 and Snail2 (formerly Snail and Slug), the Zeb family (Zeb1 and Zeb2), E47, KLF8 and Twist1 have been described to bind to CDH1 promoter, although Twist does it indirectly.[7,17] In this review we will focus on Snail, Twist and Zeb proteins, describing the post-translational mechanisms controlling their protein stability and function.

The SNAIL family

The SNAIL family of repressors is comprised of 3 members: SNAIL1 (formerly known as SNAIL), SNAIL2 (SLUG) and SNAIL3 (SMUC). It constitutes a subfamily characterized by a SNAG box in its N terminus required for transcriptional repression (**Fig. 1A**). Snail1 is the most broadly studied member of the family. Through the SNAG domain (amino acids (aa) 1-9) Snail1 recruits multiple co-repressors (such as Polycomb complex 2, Sin3A/histone deacetylases1/2 complex, Ajuba) involved in CDH1 gene repression.[19-22] Two independent Snail1 affinity

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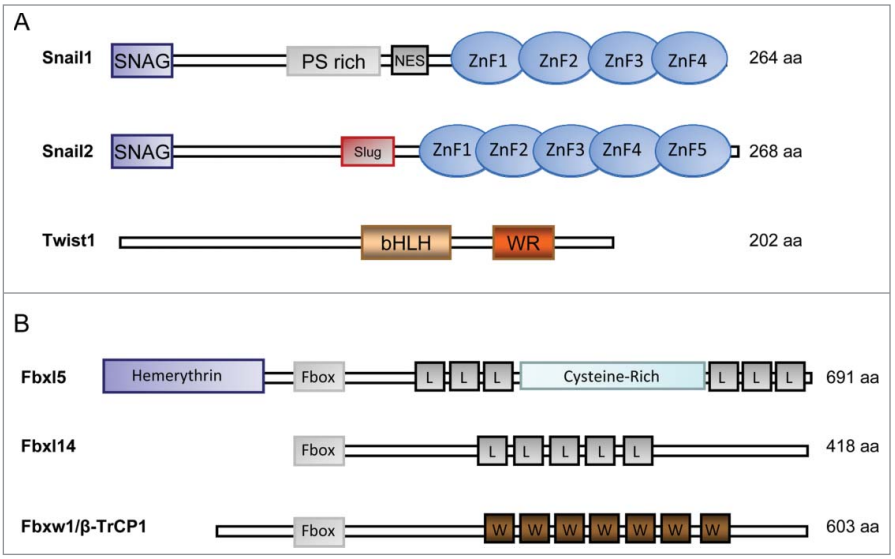

Figure 1. Schematic representation of Snail1, Snail2 and Twist1 domains (**A**) and the ubiquitin ligases involved in their degradation (**B**). (**A**) The position of the phospho-serine (PS) rich region, the nuclear export sequence (NES), the zinc finger domains (ZnF), the SLUG domain are shown for Snail1 and 2. The basic helix-loop-helix (bHLH) domain and the WR (tryptophan and arginine) motif is indicated for Twist1. (**B**) Fbxl5, Fbxl14 and Fbxw1/β-TrCP1 are represented. FBXL, F-box and Leucine-rich repeat Protein. FBXW, F-box and WD40 repeat protein. L, Leucine-rich repeat. W, WD40 repeat.

purifications coupled with mass spectrometry analysis are coincident in detecting lysine-specific demethylase (LSD1),[22,23] an enzyme that removes methyl groups from H3K4me1/2. These experiments suggest that LSD1 shows the highest affinity for the SNAG domain and many other cofactors might interact with Snail1 indirectly. In the central part Snail1 contains a phospho-serine rich domain (aa 90-120) and a nuclear export sequence (NES) located in close proximity (aa 132-143) that binds to Crm1 (Exportin-1)[24]; and at the C-terminal part of Snail1 (aa 152-264), 4 zinc fingers (ZnF) of the C2H2 type that bind DNA through E-boxes (**Fig. 1A**),[25] and a unique nuclear localization signal (NLS) mediating nuclear import.[26,27]

Snail2 (also known Slug) has been less studied than Snail1 although it also represses CDH1 and induces EMT.[28] Snail2 is imported into the nucleus through a NLS sequence similar to that observed in Snail1.[27] Snail2 has an specific central domain (named SLUG domain) capable of binding the C-terminal binding protein 1 (CtBP1) co-repressor[29] and of recruiting histone deacetylase 1 (HDAC1) (**Fig. 1A**)[28,30] A recent study shows that the co-repressors CtBP1 and nuclear receptor co-repressor 1 (NCoR) use the SLUG and SNAG domains, respectively, to be recruited to Snail2.[29] Contrary to what happens with the Snail1 knockout mouse,[31] Snail2 deleted mice are viable, showing that Snail2 is not essential for mesoderm formation.[32]

The ZEB family

The ZEB family of transcription factors is highly conserved across species and is constituted by 2 members: ZEB1 (also known as δEF1) and ZEB2 (also known as SIP1).[33] It contains 2 different domains of interaction with DNA, constituted each by 3 and 4 zinc fingers of the C2H2 and C3H type located at the N- and C-terminal part of the protein, respectively, and a central homeodomain. Zeb proteins potently inhibit CDH1 expression through the binding to CtBP co-repressors and the recruitment of HDACs and methyltransferases, Polycomb proteins, coREST and the SWI/SNF chromating remodeling protein BRG1, among other factors.[34-38] Similarly to the Snail family, TGF-β and Wnt activate these mesenchymal genes.[39,40] However, the kinetics or activation is different; for instance, Snail1 induction by TGF-β precedes and is required for Zeb1 expression. Snail1 acts at several levels: it increases Zeb1 transcription by favoring Ets1 and NF-κB translocation to the nucleus, decreases the levels of miRNA-200 targeting ZEB1 mRNA, and stabilizes Zeb1 protein.[40,41] Snail1 also induces Zeb2 protein in an indirect manner through the alternative processing of the ZEB2 mRNA.[42]

The bHLH family of transcription factors

The basic helix-loop-helix (bHLH) family of transcription factors has a common structure with 2 parallel amphipathic α-helices joined by a loop required for dimerization. bHLH proteins bind DNA as homo- or heterodimers using consensus E-boxes and can act both as transcriptional activators or repressors.[43] The 2 products of the E2A gene, E12 and E47, the inhibitory Id proteins (Id1-Id4) and the Twist proteins (Twist1 and Twist2) are members of this family, the last ones being the most widely described bHLH proteins involved in EMT.[7,44] Twist1, apart from sharing the common bHLH domain, presents at the C-terminal end of the protein a WR motif important for Twist degradation (**Fig. 1A**).[45,46] Twist represses CDH1 expression indirectly binding to this promoter and recruiting chromatin remodeling complexes containing Mi2/NuRD and SET8.[47,48]

Twist factors predominantly heterodimerize with E12 and their function is therefore determined by the availability of this protein as well as by the phosphorylation of the bHLH domain.[49] As described for Snail and Zeb family members, Twist proteins are up-regulated by TGF-β, Wnt signaling, growth factors and hypoxia. In fact Twist1 is directly modulated by low oxygen since it contains an Hypoxia Inducible Factor (HIF)-responsive element (HRE) in its promoter; this site is bound by HIF-1α in low oxygen conditions causing Twist transcription.[50]

Snail and Twist in EMT as Sensors of Cellular Stress

Expression of EMT transcription factors is controlled by many growth factors and cytokines including HGF, TGF-β or TNF-α.[5,51,52] Among these, TGF-β is the best studied EMT

inducers that cross-talks with other cell pathways, such as Wnt, Ras, Hedgehog and Notch in order to induce Snail1 and promote EMT.[53] Besides these extracelluar signals, EMT is also controlled by stress, through the activation of molecular pathways that have recently started to be analyzed.[54] For instance, hypoxia activates the EMT program with the concomitant induction of Snail1, Twist1 and Zeb2.[50,55] Snail1 expression is also induced by reactive oxygen species (ROS) that increases both SNAIL1 mRNA and protein stability.[56-58] Curiously, Snail1 expression *per se* also activates the ROS pathway.[57,59] Genotoxic stress caused by DNA damage also induces Snail1 and Snail2 expression, a response that has been attributed to a cellular mechanism to avoid programmed cell death.[60] Other results also link stress, the EMT phenotype and the acquisition of chemo-resistance, and are associated to the up-regulation of Snail1/2 or Twist1[61-64] and the acquisition of some cancer stem cell-like properties.[65] For instance, cellular stress mediated by ionizing radiation promotes EMT, stimulating invasion, metastasis and radio-resistance of tumor cells[66]; this insult up-regulates Snail1, at least in part due to protein stabilization,[67-69] and Snail2, increasing its transcription[70]; consequently transcription of the pro-apoptotic genes Puma[70] or PTEN[71] are down-regulated and cells become more resistant to apoptosis.[72]

Ubiquitin Ligases Controlling Snail and Twist Stability

Snail and Twist are short-lived proteins since they are rapidly polyubiquitinated and degraded by the 26S proteasome system in normal cells. Polyubiquitination is a process by which the 76-amino acid protein ubiquitin is attached to a target protein. Three types of enzymes are the ones responsible for the final ubiquitination of a substrate: first the Enzyme 1 (E1) or ubiquitin-activating enzyme activates the ubiquitin molecule in an ATP (adenosine triphosphate)-dependent manner; then a second enzyme (E2) or ubiquitin-conjugating enzyme transfers the activated ubiquitin moiety directly onto the substrate or to a third enzyme (E3) called ubiquitin ligase.[73,74] The way in which ubiquitin is loaded onto the substrate depends on the specificity of the E3. These enzymes belong to 2 grand subfamilies: the RING/RING-like or the HECT types. To date, only E3 ligases of the RING-type have been described to be relevant in mediating Snail and Twist ubiquitination. Degradation of most EMT factors is strictly dependent on multi-subunit RING-type E3s and include the so-called SCF (Skp1-Cullin1-F-box) complex containing a Cullin1 (Cul1) scaffolding protein, the adaptor protein Skp1, the RING-finger protein Rbx1/Roc1 and an F-box protein[75] (**Fig. 2**). The F-box protein is the substrate-recognition subunit and contains, as its name implies, an F-box domain, a 50 amino acid motif used to bind Skp1 linking the N-terminal part of Cullin to the F-box protein. Moreover, Rbx1 binds to the C-terminal part of the Cullin and acts as a docking site for the ubiquitin-activated E2 protein[76,77] (**Fig. 2**). So far, 4 different RING-type E3s ubiquitin ligases have been described to be relevant in EMT: 3 SCF-E3s named SCF-β-TrCP1, SCF-Fbxl14 and SCF-Fbxl5; and one single-subunit, Mdm2.

SCF-β-TrCP1/Fbxw1

Fbxw1, commonly named β-TrCP1 (β-transducin-repeat containing protein), is the first member of a F-box subfamily containing a specific domain characterized by the presence of WD40 repeats (this subfamily is commonly named as FBXW and comprises 10 members in humans) (**Fig. 1B**).[78] Proteins that are targeted by the ubiquitin-ligase machinery contain a degradation signal or degron, which is defined as a minimal element

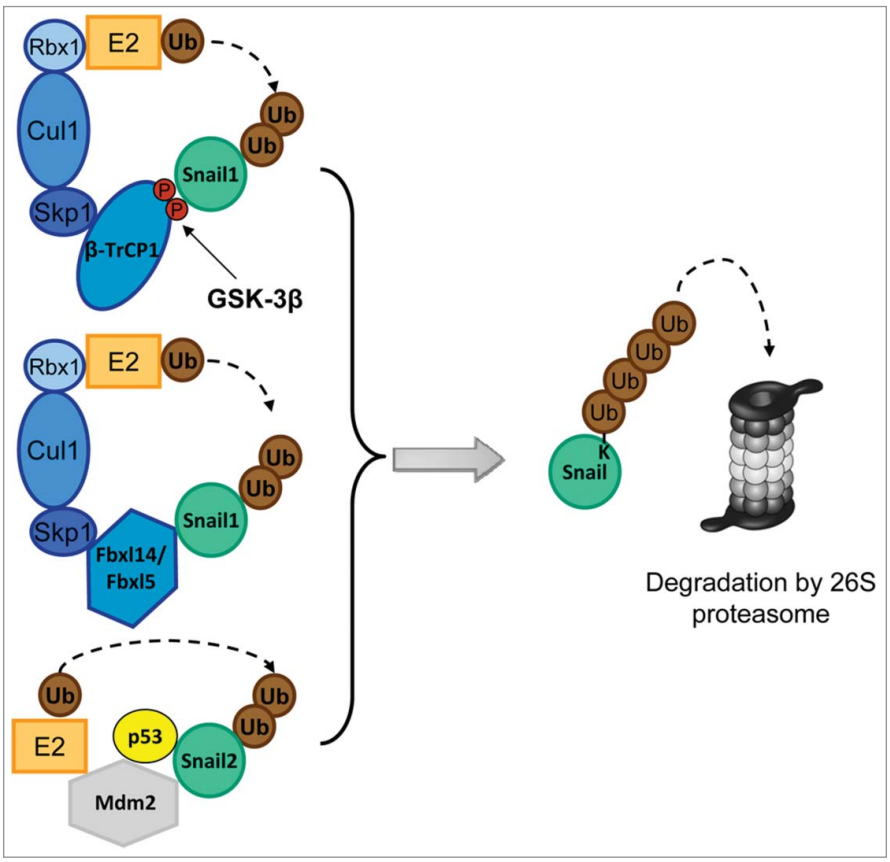

Figure 2. Composition of the E3 complexes targeting Snail1 and Snail2. The Skp-1-Cullin-1-F-Box (SCF) E3s, SCF-Fbxl14 and SCF-Fbxl5 are multimeric E3 ligases that mediates the ubiquitin (Ub) transfer from the E2 conjugating enzyme to Snail. SCF E3s are composed of the scaffold protein Cullin1 (Cul1), which interacts with Skp1 and the RING-finger protein Rbx1. The substrate binding affinity is mediated by the F-box protein (β-TrCP1, Fbxl14 and Fbxl5). The SCF-β-TrCP1 binds Snail1 only when double-phosphorylated by GSK-3β. Mdm2 is a single-subunit RING-finger E3 that targets Snail2 when p53 is bound.

within a protein sufficient for recognition and degradation by the proteolytic apparatus.[79] In the case of Snail1, the β-TrCP1 degron is a phosphorylated sequence (commonly named phospho-degron) $DpS^{96}GxxpS^{100}$, consequence of the action of GSK-3β on Snail1 residues S96 and S100 (**Fig. 2 and 3**).[80] This consensus motif for β-TrCP1-mediated degradation is similar to that previously described on β-catenin.[81] More than 35 substrates have been described to contain a similar sequence for β-TrCP1/2 (β-TrCP2 is also known as Fbxw11), all requiring the participation of different kinases.[82]

Besides being potently upregulated by proteasome inhibitors, Snail1 protein stability is also enhanced by lithium ion (Li^+), an inhibitor of GSK-3β.[80] Phosphorylation by this enzyme of the Ser-rich Snail1 domain is relevant not only for degradation but also for nuclear export.[24] Phosphorylation on residues S104, S107 and possibly other Serines between S107 to S119 promote Snail1 localization to the cytoplasm,[80,83] uncovering a nuclear export sequence.[24] Moreover, phosphorylation of Ser104 acts as priming site for the second round of phosphorylations on S96 and S100 that takes place in the cytosol and originates the above described consensus destruction motif or degron $DSp^{96}GxxpS^{100}$ recognized by β-TrCP1[24,80] (**Fig. 3**). Snail2 also contains 2 GSK-3β motifs inside the Slug domain in a recently identified Serine rich region that alike Snail1 affects ubiquitination and degradation by β-TrCP1, although this sequence does not contain the classical β-TrCP1 destruction motif.[84,85]

Besides phosphorylation of the substrate, an active SCF-β-TrCP1 complex requires the neddylation (incorporation of the small protein Nedd8) of Cul1 by a Nedd8-E3 ligase.[86] Interestingly, TNF-α promotes the induction of the COP9 signalosome 2 protein (CSN2) that removes Nedd8 (or reverses neddylation) from cullins increasing Snail1 stabilization during inflammation.[87] As a consequence of this, TNF-α reduces the association of Snail1 with β-TrCP1 complex due to misassembly and, surprisingly, the phosphorylation of Snail1 by GSK-3β.[87]

β-TrCP1(−/−) mice have been generated; these animals develop normally and only males present a mild phenotype consisting in reduced fertility due to the accumulation of metaphase I spermatocytes in testes.[88] This is consequence of the stabilization of the β-TrCP1 substrates cyclin A, cyclin B, and Emi1 but not β-catenin.[88,89] Curiously, at least in MEFs, β-catenin stabilization requires additional silencing of β-TrCP2[88] although this ubiquitin ligase does not interact with β-catenin.[90] Similarly to β-catenin, Snail1 is stabilized upon the combined depletion of both ligases, obtained by expression of an sh-β-TrCP2 in β-TrCP1 knockout mice.[89] These double-deficient mice are normal although show a more severe testicular phenotype than the β-TrCP1(−/−) mice with absence of spermatids and meiotic cells. Interestingly, this phenotype is reversed upon Snail1 depletion.[89] These results suggest that β-TrCP1 and β-TrCP2 are redundant in controlling the stability of Snail1 and β-catenin, although, as happens for β-catenin, β-TrCP2 expression does not induce Snail1 degradation.[91] Therefore, more work is needed to understand whether the 2 homologues play an individual, synergistic, or redundant role in controlling Snail1 and β-catenin stability under different experimental conditions.

SCF-Ppa/Fbxl14

Fbxl14 is the F-box subunit of the SCF complex and the human homolog of Partner of Paired (Ppa) gene product.[78] This protein degrades Snail2 (and Snail1) in *Xenopus laevis* in a phosphorylation-independent manner.[92] FBXL14 belongs to the FBXL subfamily of F-box proteins formed by 21 members, all of them containing Leucine-rich repeats (LRR) at the C-terminus (5 LRRs in the case of FBXL14) (**Fig. 1B**). There are 2 homologues of Fbxl14 in zebrafish, namely Fbxl14a and Fbxl14b that are differently regulators of dorsoventral patterning through the targeting of the MAP kinase phosphatase-3 (Mkp3).[93] Ppa is highly conserved across species,[94] in fact, the SCF-Fbxl14 ubiquitin ligase also targets Snail1 in mammalian cells (**Fig. 2**).[91] Interestingly, Fbxl14 and β-TrCP1 modify the same lysines in Snail1: K98, K137 and K146, all of them located in the N-terminal part[91] (**Fig. 3**). Lander *et al.* have suggested a general role of Ppa/Fbxl14 in EMT since it promotes the degradation of other transcription factors involved in this process such as Twist1 and Zeb2.[46] This last finding is intriguing because Snail, Zeb and Twist proteins are not structurally related. In the case of Snail2 the Ppa/Fbxl14 interaction region is an hydrophobic sequence of the N-terminus comprising amino acids 31 to 64, with

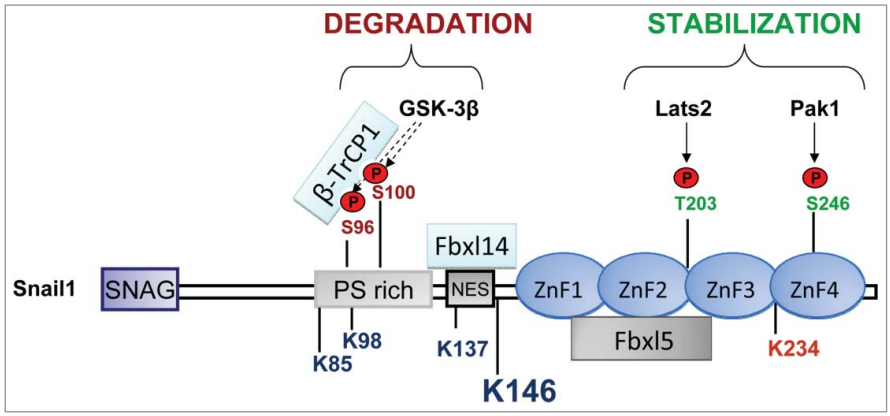

Figure 3. Snail1 phosphorylation sites linked to stabilization or degradation. Stabilizing phosphorylation sites are mainly localized in the C-terminus except the stabilizing ATM phosphorylation site in S100 and ERK2 phosphorylation sites in S82 and S104 (not indicated). The corresponding kinases are indicated by arrows. Phosphorylation sites promoting degradation are in the N-terminus (GSK-3β). The interaction with the F-box proteins Fbxl14 and β-TrCP1 is located in the N-terminus and with Fbxl5 in the C-terminus. The Lysines (K) that are ubiquitinated are indicated: K85, K146 and K234, were found to be specifically ubiquitinated by SCF-Fbxl5 and K98, K137 and K146 were modified by SCF-Fbxl14 or SCF-β-TrCP1. Although other K can be also modified, K146 is the best substrate of the 3 ligases and is the major ubiquitination site. K234 is marked in red because its modification by SCF-FBXL5 has a non-degradative role by decreasing DNA-affinity.

low homology with Snail1 and no similarity with the Twist interacting sequence located on the C-terminal WR domain.[46,92] In the case of Snail1 the precise binding sequence has not being totally determined although it comprises amino acids 120 to 151[91] (**Fig. 3**). This sequence is also rich in hydrophobic amino acids suggesting that Fbxl14 interaction is dependent on the presence of hydrophobic subdomains in their substrates.

Interestingly, Fbxl14 is sensitive to stress conditions and is specifically downregulated by hypoxia.[91] This decrease happens at the transcriptional level at is dependent on the elevated expression of Twist1 protein in hypoxic cells. Therefore, hypoxia down-regulates the expression of FBXL14 causing the stabilization of several EMT core transcription factors, such as Snail1 and Twist1. This causes a feedback loop in which the protein stability of these transcription factors, normally short-lived, is highly increased. Analysis of human colon tumor samples shows an inverse correlation between hypoxia and FBXL14 levels.[91] Other authors have shown that hypoxia causes inhibition of GSK-3β through the intracellular generation of ROS, as determined by analyzing the phosphorylation of Ser9, a modification associated to loss of function of this kinase.[95] Our unpublished results also indicate that hypoxia-induced FBXL14 down-regulation is in some cells accompanied with GSK-3β inactivation (Viñas-Castells R, unpublished). It is likely that a complete EMT requires the inactivation of both ubiquitin ligases achieved through GSK-3β inhibition and Fbxl14 down-regulation, suggesting a synergistic effect between different EMT-inducing signals.

In vivo depletion of Ppa/Fbxl14 has been performed in *Xenopus* embryos, evidencing a relevant role for Ppa in neural crest development[92]; other experiments in zebrafish embryos indicate that Fbxl14 regulates axis formation.[93] Unfortunately, knockout mice models for FBXL14 have not been developed yet.

SCF-Fbxl5

A shRNA screening for Snail1-specific F-box E3 ubiquitin ligases resulted in the identification of Fbxl5.[69] Besides comprising a hemerythrin domain capable of binding iron in its N-terminal part, Fbxl5 contains an F-box domain and 6 LRRs (**Fig. 1B**).[96,97] This ligase is mainly, but not exclusively, localized in the nucleus and polyubiquitinates Snail1 acting on several lysines (**Fig. 2 and 3**): K85 and K146, both placed in the N-terminus, as well as K234, located in the Snail1 C-terminal domain. Different to Fbxl14 or β-TrCP-1, Fbxl5 interacts with this Snail1 domain (ZnF1 to ZnF3)[69] (**Fig. 3**). Since K146 (located in the N-terminal part) is the main substrate of this ubiquitin ligase, FBXL5 requires 2 different domains of Snail1 protein for degradation. Moreover, K234 ubiquitination by FBXL5 totally impairs Snail1 interaction with the DNA, indicating an unexpected role for this modification in controlling DNA binding affinity and also showing another significant difference with respect to β-TrCP1 or Fbxl14.[69] Although Snail1 ubiquitination by SCF-Fbxl5 takes place in the nucleus, the protein is degraded in the cytosol. Accordingly, inhibition of Snail1 export using Leptomycin B prevents Snail1 degradation by Fbxl5. Therefore, Snail1 is not targeted for degradation in the nucleus, and nucleocytoplasmic shuttling is essential for degradation, an effect also required for p53 efficient proteolysis.[98] It remains to be established why nuclear proteasomes do not process Snail1[99]; it is possible that the unfolding of the protein caused by ubiquitination promotes its rapid interaction of the NES with Crm1 exporter and its exit from the nucleus before it can be proteolyzed. Alternatively, Fbxl5 might produce an incomplete ubiquitination that needs to be extended by cytoplasmic ubiquitin ligases. According to our model, Fbxl5 ubiquitination, besides decreasing Snail1 interaction with target promoters, unfolds the molecule exposing the NES facilitating Snail1 nuclear export. Once Snail1 is exported, the coordinated effects of cytoplasmic F-box proteins β-TrCP1 and Fbxl14, and maybe other ubiquitin ligases, may cooperate and complete ubiquitination. These results also have implications in the regulation of Snail1 protein half-life since Fbxl5-induced degradation should be impaired by all the post-translational modifications preventing nuclear export.

Fbxl5 is a more specific E3 ubiquitin ligase than β-TrCP1 or Fbxl14, which control the stability of multiple EMT factors and even of other proteins outside this pathway. When analyzed, Fbxl5 degrades Snail2 but not Twist1 or Zeb1. This is probably related to the fact that, contrary to other ubiquitin ligases that interact with the central domain, Fbxl5 binds the C-terminal region of Snail1; therefore it will only tackle transcription factors with a similar zinc finger domain, such as Snail2.[69]

Similarly to Fbxl14, Fbxl5 expression is decreased by some conditions that induce Snail1 stabilization. Besides being destabilized by iron depletion, FBXL5 is potently down-regulated after γ-irradiation (IR), a cellular stress condition also causing a complete or partial EMT.[69] This suggests that Fbxl5 might promote radio-sensitivity of cancer cells. The down-regulation of Fbxl5 by IR depends on both the decrease in its mRNA and the destabilization of the protein mediated by the same hemerythrin domain that confers sensitivity to iron.[69] The mechanism linking IR with hemerythrin-dependent destabilization of Fbxl5 is still unknown, but it is likely that IR might indirectly reduce the iron levels required for hemerythrin stability[100] or modulate the levels of the ubiquitin ligase that targets Fbxl5.[101] Moreover, Fbxl5 is also downregulated by hypoxia[97] which can also contribute to the Snail1 stabilization observed in these conditions.

The *in vivo* role of FBXL5 in EMT using knockout mice has not been determined yet. This is probably due to the fact that FBXL5, apart from its role in EMT, is a master regulator of iron homeostasis and its depletion is embryonically lethal, making difficult to study its role on other substrates such Snail1.[102] FBXL5-null mice die *in utero* due to the stabilization of the iron-response protein-2 (IRP2), a RNA binding protein that inhibits translation of ferritin mRNA and blocks transferrin receptor mRNA degradation. As a consequence of IRP2 stabilization, there is a general and lethal iron overload. Interestingly, FBXL5 −/−IRP2−/− mice do not show any phenotype; it is possible that these mice respond to signals activating Snail1 with a higher upregulation of this factor.

Mdm2

Another ligase capable to degrade the Snail family member Snail2 is Murine Double Minute 2 (Mdm2, or Hdm2 in humans). The main role of Mdm2 consists in the ubiquitination and degradation of the tumor suppressor p53, which prevents cancer progression by inhibition of proliferation and induction of apoptosis.[103,104] Curiously, p53 can antagonize the action of the EMT transcription factor Snail2 by forming a p53-Snail2-Mdm2 complex that facilitates the degradation of Snail2 (**Fig. 2**).[105] Due to the structural similarities between Snail2 and Snail1 it is possible that the same ligase may target both proteins. In fact, Mdm2 can facilitate Snail1 degradation in a p53-dependent manner,[106] although the precise mechanism has not been elucidated yet. Another link evidencing a p53-Snail1 antagonism has been proposed by Weiss and coworkers that have shown that p53 transactivates miRNA-34, which suppresses SNAIL1 mRNA by binding to its 3′-UTR.[107] p53 also regulates EMT by transactivating miR-200b and c targeting ZEB1/2 mRNA.[108]

An intriguing question is whether the Mdm2-mediated Snail1/2 degradation takes place in the nuclear or cytoplasmic compartment. The fact that p53 ubiquitination by Mdm2 may occur in the nucleus but efficient degradation requires nuclear export,[109] suggests that Snail1/2 degradation may follow the same pattern, as previously commented for Fbxl5. Thus, Mdm2 might cooperate with Fbxl5 facilitating Snail1/2 nuclear ubiquitination and subsequent export and cytoplasmic degradation by the SCF-β-TrCP1 or SCF-Fbxl14 ubiquitin ligases. Another interesting link between p53 and EMT is mediated by Twist1 that interacts with the C-terminal part of p53 promoting its degradation by Mdm2.[110] Since p53 negatively control Snail1 expression at several levels (see above), induction of Twist might upregulate Snail1 by facilitating p53 degradation, further demonstrating the complex network that interconnects the expression of these 2 transcriptional factors. Finally, adding more complexity to the system, Mdm2 is itself a substrate of β-TrCP1 upon its phosphorylation by CK1 after DNA damage.[111] Therefore, it is likely that the genotoxic stress-dependent degradation of Mdm2 might contribute to Snail stabilization.

Mouse knockout models for p53 or Mdm2 do not display EMT defects. However, the loss or mutation of p53 that commonly occurs in cancer have been shown to have a strong impact in EMT, likely due to the central role of p53 antagonizing Snail1 or 2 expression, as described above.[105,107]

Control of Snail1 Protein Stability by Post-Translational Modifications

Phosphorylation

Snail1 undergoes phosphorylation by several protein kinases affecting its half-life. The first group of these kinases promotes its degradation; the paradigm is GSK-3β that, as indicated before, is required by the action of SCF-β-TrCP1.[80,85] The catalytic action of this kinase on Snail1 is controlled by direct inactivation by PKB/AKT phosphorylation on Ser9,[80] and by the chaperone Axin2, that promotes GSK-3β nuclear export.[112] Therefore, Axin2 prevents the phosphorylation of Snail1 in the nucleus, maintaining this protein in this compartment and precluding its degradation not only by β-TrCP1 but by the cytosolic Fbxl14 as well.[112] Axin2 expression is controlled by the Wnt canonical pathway since Axin2 promoter contains β-catenin-TCF4 elements, linking Wnt activation with Snail1 upregulation and EMT.[112] Another interesting mechanism stabilizing Snail1 through GSK-3β inactivation is triggered by prostaglandin that promotes the interaction of this protein kinase with the receptor-coupled G protein βγ subunits.[113]

GSK-3β phosphorylation needs to be primed. Several protein kinases have been described to be able to do it, such Casein kinase 1 epsilon (CK1-ε) that interacts with the zinc finger domain of Snail1,[114] CK2 (an hetero-tetramer composed of 2 catalytic subunits, α/α′, and a dimer of regulatory β subunits),[115] and DYRK2.[116] Interestingly, both CK2β and DYRK2 are downregulated in breast cancer patients what would prevent Snail1 phosphorylation, and increase Snail1 levels and subsequent EMT.[115,116] Finally, Protein kinase D1 (PKD1) has also been reported to modulate Snail1 function, by phosphorylating S11 and promoting its nuclear export triggered through 14-3-3σ binding.[117] Moreover, PKD1 inhibits the activity of DNA-bound Snail1, likely preventing its interaction with co-repressors.[118] However, these results have to be confirmed since other authors have demonstrated that PKD1 phosphorylation enhances nuclear Snail1 transcriptional repression activity.[119]

The group of kinases promoting Snail1 stabilization is also abundant. PKA and CK2α phosphorylate Snail1 on S11 and S92, respectively, increasing Snail1 stability and repression of E-cadherin.[120] Thus, the role of CK2 is unclear as indicated above it has been reported to induce Snail1 degradation.[115,120] Other stabilizing phosphorylations affect residues in the C-terminal part of Snail1 and are catalyzed by 4 different kinases (**Fig. 3**): Lats2, p21 activated kinase 1 (PAK1), the Ataxia telangiectasia mutated (ATM) kinase and ERK2. Lats2 phosphorylation on Snail1-T203 is activated during TGF-β induced EMT and potentiates Snail1 activity by promoting nuclear retention.[121] Similarly, PAK1 phosphorylates Snail1 on S246 enhancing its nuclear abundance and repressive potential.[122] PAK1-dependent Snail1 phosphorylation on S246 is up-regulated in response to IR, an insult that strongly increases Snail1 stabilization (see above). In agreement with the notion that the DNA damage response may enhance tumor progression, both IR and the topoisomerase I poison camptothecin promote Snail1 phosphorylation on S100, which is catalyzed by the ATM kinase. Surprisingly, although S100 is also modified by GSK-3β inducing degradation, in the context of DNA damage phosphorylation of this residue prevents the interaction with GSK-3β and stabilizes Snail1.[68,80] This has been related to the action of HSP90 that in these conditions binds to this phosphorylated residue precluding GSK-3β action on S96 and retaining Snail1 in the nucleus.[68] Finally, DDR2 (discoidin domain receptor 2) activation regulates Snail1 stability by stimulating ERK2 and phosphorylating Snail1 on S82 and S104, which also leads to Snail1 nuclear accumulation and increased protein half-life.[123] Therefore, it is likely that most of these modifications promote nuclear

retention facilitating the interaction with nuclear chaperones, as it previsouly described for HSP90 and inhibiting binding to Snail1 of Crm1 nuclear exporter.[68] Accordingly, another chaperone, HSP27 expressed during fibrosis and in prostate and breast cancer binds to and stabilizes Snail and consequently induces EMT.[124-126]

A recently described unexpected role for GSK-3β consists in controlling Twist stability and the interaction between Snail1/2 and Twist.[127] Twist is phosphorylated by GSK-3β in the WR domain and this phosphorylation renders Twist less stable.[127] However, in cells co-expressing Snail1, Twist is protected from destabilization. The proposed mechanism suggests that Snail1 binds to Twist and interferes with its interaction with Ppa/Fbxl14 ubiquitin ligase. Snail1-Twist association is potentiated by Twist1 phosphorylation by GSK-3β that promotes the interaction with the C-terminal zinc fingers of Snail1/2 and causes the loss of function of Snail1 since its recruitment to E-box sequences is impaired.

The role of other kinases on Snail2 and Twist1 stability has been less studied. Besides GSK-3β, Snail2 is phosphorylated on serines 4 and 88 to enhance its repressor activity of CDH1 expression and the induction of EMT.[29] Twist may also be phosphorylated by CK2 on S18 and S20, or by MAPKs on S68, upon IL-6 or TGF-β stimulation, respectively, resulting in Twist1 protein stabilization.[128,129] Finally, Snail1 phosphorylation by GSK-3β is reversed by the small C-terminal domain phosphatase (SCP), which interacts with Snail1 in the nucleus and prevents its nuclear export and degradation.[130]

Other post-translational modifications

Snail1 can be poly(ADP-ribosyl)ated by poly(ADP-ribose) polymerase 1 (PARP1), causing its stabilization.[131] Another dynamic modification occurs on S112 Snail1 and consists of its modification by β-N-acetylglucosamine (O-GlcNAc). This modification prevents protein phosphorylation by GSK-3β and is a consequence of the activity of the O-GlcNAc transferase activated in hyperglycemic conditions. Therefore, glycosylated Snail1 cannot be phosphorylated by GSK-3β what enhances Snail1 action and provides a link between glucose metabolism and the control of EMT.[132] Another relevant modification of EMT-inducing factors consists in Twist diacetylation that allows recruitment of BRD4, activation of the WNT5A promoter and the enhancement of EMT properties of basal-like breast cancer cells.[133] Finally, factors inducing EMT are also sumoylated. So far only Zeb2 has been reported to be sumoylated on K391 and K866 by the polycomb protein Pc2, attenuating E-cadherin repression.[134] Because inhibition of the sumoylation enzymes by TGF-β is relevant for the action of this cytokine in triggering EMT,[135] it is important to elucidate whether sumoylation affects other EMT transcription factors.

Conclusion

Different reports indicate that Snail and Twist protein levels are rapidly upregulated in response to cellular stress as a survival mechanism of cancer cells. We have reviewed here the ubiquitin ligases acting on Snail and Twist and how they are regulated by different mechanisms including phosphorylation by different kinases. However, the E3 ligases controlling other transcriptional factors relevant for EMT, such as Zeb1 or 2, are practically unknown.[46] Future work is also required to determine if additional E3 ligases may have a role in controlling Snail1 or Snail2 stability. For example, it has been recently reported that Slug could also be ubiquitinated by CHIP, a U box-containing E3 ubiquitin ligase, upon phosphorylation by GSK-3β.[136] It is also probable that alternative mechanisms for stabilization of these EMT transcription factors exist. For example, one unexplored field is the role of deubiquitinases (DUBs), isopeptidases that specifically remove polyubiquitin chains from substrates stabilizing them.[137] There are not examples of DUBs involved in EMT, besides the Ubiquitin Specific Protease-1 (USP1) that deubiquitinates and stabilizes Id proteins essential for the maintenance of the mesenchymal phenotype and prevention of osteoblastic differentiation.[138] Another DUB called ubiquitin C-terminal hydrolase-1 (UCH-L1) promotes EMT but the target substrates are not known.[139] It is probable that one or several DUBs enhance Snail, Twist and Zeb stability under pathological conditions. Because these DUBs are putative drugable entities, their future characterization will allow the generation of new inhibitors to block the EMT process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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